Consumer and Corporate Affairs Canada | Consommation et Corporations Canada

(11) (A) No. 1 202 584

(45) ISSUED 860401

(52) CLASS 195-35.2

(51) INT. CL. C12N 11/02

(19) (CA) **CANADIAN PATENT** (12)

(54) Proteases Fixed on a Carrier and Their Use in Biotechnology

(72) Burger, Erich;
Hartmeier, Winfried,
Germany (Federal Republic of)

(73) Granted to Boehringer Ingelheim International G.m.b.H.
Germany (Federal Republic of)

(21) APPLICATION No. 415,462

(22) FILED 8211.12

(30) PRIORITY DATE Germany (Federal Republic of)
(P 31 45 684.7) 811119

No. OF CLAIMS 11 - NO DRAWING

Canada

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CCA-274 (11-82)

The invention relates to proteases covalently fixed on a carrier protein in a novel manner, the preparation thereof and the use thereof in biotechnology.

Proteases are of considerable importance in the manufacture of drinks since protein turbidity may occur in many clear drinks (such as wine, beer and fruit juices) as a result of heating or cooling.

One of the present approaches to solving this problem is treatment with bentonite. This certainly achieves the required stability but at the same time there are a number of serious disadvantages. Thus, the strong adsorptive effect also eliminates a number of valuable substances such as aromatic substances, bouquet and/or dyestuffs, and moreover some undesirable metal ions may be introduced, and thirdly considerable quantities of sediment are produced which are laborious to process.

It has therefore already been proposed that the proteins which cause cloudiness under the effect of heat or cold be decomposed by means of proteases (vide Z. für Lebensmitteluntersuchung und -forschung 134 (1967), pages 87-97).

However, there are disadvantages with this method too. First, the costs of enzyme enrichment and isolation are extremely high since the enzymes are unstable and cannot be recovered either. Moreover, it is inadvisable or even illegal according to the laws on foodstuffs to have any soluble enzymes remaining in the drink.

One way of solving these problems is to make the enzymes used insoluble by fixing them on carrier substances which are insoluble in water. This enables the enzymes to be recovered and

re-used several times.

A number of methods of fixing enzymes are already known. A synopsis of the latest results in this field is provided, for example, by J.C. Johnson (Editor) in "Immobilized Enzymes, Preparation and Engineering", Noyes Data Corp., Park Ridge, N.J., USA (1979).

The fixing of the enzymes on carriers results in other advantages in addition to those mentioned above. These include, for example:

a continuous method of operation

increased stability

a smaller reaction volume

no contamination of the reaction mixtures

no heat precipitation of the enzyme.

There is no standard process for bonding an enzyme to an insoluble carrier substance. In order to reach the objective of obtaining maximum biological activity of the enzyme, the carrier substance and method of immobilization must be individually matched to the enzyme.

Particularly preferred carrier substances for this purpose (on the grounds that they are easy to process and inexpensive) are gel-forming proteins such as gelatine or fresh egg white (see for example German Auslegeschrift 22 46 002). However, these carrier substances are not suitable for fixing enzymes with a proteolytic activity since they are hydrolysed by them.

It has therefore already been proposed (see German Auslegeschrift 26 36 206.1-41) that the gel-forming proteins be

denatured by chemical hardening (e.g. with formaldehyde, glutardialdehyde or diisocyanate) in order to make them safe from attack by the proteases fixed on them. In this way, rennin or even pepsin, for example, may be fixed on a carrier (vide Biotechnology Letters 1 (1979) pages 225-230).

Surprisingly, it has now been found that proteases can also be fixed to gel-forming proteins even if the proteins are not subjected to a complex preliminary chemical treatment.

The invention therefore relates to a process for immobilizing proteases on gel-forming proteins which is characterised in that the protease is mixed with gel-forming protein (e.g. gelatine, egg white, soya protein and the like, preferably albumin), the mixture is treated with a water-miscible organic solvent (precipitating agent) and subsequently the resulting particle suspension is reacted with difunctional and/or polyfunctional cross-linking agents. The invention further relates to the enzyme preparations fixed on a carrier which are obtained in this way and their use in decomposing proteins.

The mixture of proteolytic enzyme and gel-forming protein may be prepared by dissolving or suspending the enzyme in an aqueous solution of the gel-forming protein. The temperature is preferably adjusted so that the active mixture is obtained in liquid form.

The mixture of proteolytic enzyme and gel-forming protein is preferably prepared by first dissolving the proteolytic enzyme (e.g. pepsin, papain, etc) in water and then mixing it with an aqueous solution of the gel-forming protein. This mixture is then combined with an organic solvent which has an enzyme-precipitating

activity and is compatible with the enzyme. Examples of such a solvent include acetone, ethanol, isopropanol and butanol. The particle suspension formed by the precipitating agent is allowed to react with difunctional or polyfunctional cross-linking agents in such a manner and for such a time that the mixture of proteolytic enzyme and gel-forming protein is cross-linked as completely as possible. The particulate mixture may also be separated from the organic liquid beforehand and only then treated with the cross-linking agent.

For the cross-linking reaction, difunctional and polyfunctional cross-linking agents for proteins may be used. Preferably, an aqueous solution of glutaraldehyde is used. It is also possible to dissolve the coupling reagent in one of the enzyme-precipitating liquids mentioned above and add it in this form to the aqueous mixture of proteolytic enzyme and gel-forming protein.

This reaction mixture consisting of proteolytic enzyme, gel-forming protein, enzyme-precipitating liquid and cross-linking agent is vigorously stirred for some time, preferably from 0.5 to 4 hours. The coupling reaction is usually carried out at ambient temperature.

After the bonding reaction has ended, the preparation is washed several times, preferably with water. The product thus obtained can then be dried to give the required water content (e.g. by spray drying or drying with acetone).

The enzyme products prepared by this procedure may be used in batch processes for the proteolysis of protein (protein degradation), with the enzyme product being separated off and re-

used, or in continuous processes in an enzyme reactor. They are particularly suitable for treating protein-containing beverages (such as wine, beer and fruit juices) where the end product should not contain any residual enzymes.

The proteolytic enzyme used is preferably pepsin. The pepsin activity of both the soluble form and the immobilised form is determined by the Anson method (Rick,W & Fritsch, W.-P. in Bergmeyer (Editor): Methoden der enzymatischen Analyse, 3rd edition, volume 1, pages 1086-1090, Verlag Chemie, Weinheim, 1974).

The following Examples serve to illustrate the invention:

Example 1

2 kg of pepsin were dissolved in 10 l of distilled water at ambient temperature. This enzyme solution was mixed with an aqueous albumin solution (200 g of albumin in 5 l of distilled water). The resulting suspension was added to 24 l of isopropanol (80%) with stirring, at a temperature of 25°C, and then 1 litre of glutardialdehyde (25%) was added thereto. This reaction mixture was stirred at 25°C for 2 hours. At the end of this time, the reaction product was separated by centrifuging and thoroughly washed with 200 l of water. The washed product was dried using a laboratory spray drier with a 2-component nozzle with an air inlet temperature of 155°C, an air exit temperature of 75°C and an air throughput of 420 Nm^3/h. The pepsin used (20 kg) had a total starting activity of 6000 AE (Anson units). The dry preparation fixed on a carrier amounted to 415 g and had a total activity of 3800 AE, corresponding to a recovery of 63.3% after immobilization and spray drying.

Example 2

20 g of pepsin (5 AE/g) obtained from pigs' stomachs were dissolved with 2 g of egg albumin in 150 ml of distilled water. 200 ml of ice-cold acetone were stirred into this solution. After some minutes, 10 ml of a 25% glutardialdehyde solution were added to this suspension and the resulting mixture was stirred or shaken at ambient temperature. After a reaction period of about 2 hours, the reaction product was filtered off and thoroughly washed with distilled water. The filtrate was suspended in ice-cold acetone. The immobilised enzyme product was separated from the acetone by filtration and then the filtrate was dried at 45°C in a vacuum-type drying cupboard. 2.5 g of dry preparation fixed on a carrier were obtained, with an activity of 11 AE/g.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. Process for the preparation of proteases fixed on a carrier of gel-forming proteins, characterised in that the proteases are mixed with a gel-forming protein, the mixture is treated with an organic solvent having an enzyme-precipitating activity and subsequently the particle suspension formed is reacted with one or more cross-linking agents.

2. Process as claimed in claim 1, characterised in that albumin is used as the gel-forming protein.

3. Process as claimed in one of claims 1 or 2, characterised in that the protease is pepsin.

4. Process as claimed in claim 1, characterised in that glutardialdehyde is used as a cross-linking agent.

5. Process as claimed in claim 1, characterised in that the protease is pepsin, the gel-forming protein is albumin, and the cross-linking agent is glutardialdehyde.

6. Protease preparation fixed on a carrier, characterised in that the protease is covalently bonded, by means of a cross-linking agent, to a non-hardened gel-forming protein.

7. Protease preparation as claimed in claim 6, characterised in that pepsin is covalently bonded by means of glutardialdehyde to a gel-forming protein.

8. Protease preparation as claimed in claim 7, characterised in that the gel-forming protein is albumin.

9. A method of removing residual enzymes from protein-containing fluids which comprises treating the fluid with a protease fixed on a carrier according to claim 6.

10. Method according to claim 9 wherein the fluid is a beverage.

11. Method according to claim 10 wherein the fluid is wine or beer.

FETHERSTONHAUGH & CO.

OTTAWA, CANADA

PATENT AGENTS

ABSTRACT

Proteases fixed to non-hardened gel-forming proteins by covalent cross-linking and their use for removing protein turbidity from drinks such as wine, beer or fruit juices.